AMENDMENTS TO THE SPECIFICATION

Please add the following new section heading at page 1 after the CROSS-REFERENCE TO RELATED APPLICATIONS section:

FIELD OF THE INVENTION

Please add the following new section heading at page 1, line 7, as follows:

BACKGROUND OF THE INVENTION

Please amend the paragraph beginning at page 1, line 7, as follows:

In the food industry a growing trend toward "light" and ~~low-calory~~ low calorie can be observed. The use ~~herein~~ of too much fat and/or sugar in products is avoided. To nevertheless be able to provide food products with a sweet taste, an increasing number of sugar substitutes are becoming commercially available. Aspartame is a known example thereof. Aspartame, however, has poor organoleptic properties.

Please amend the paragraph beginning at page 1, line 14, as follows:

Another type of sugar substitute is formed by oligosaccharides. Oligosaccharides are molecules which consist of two or more monosaccharides such as fructose and/or glucose. The monosaccharides in ~~the said~~ oligosaccharides are linked to each other either by β-(2-1)[[-]] or by β-(2-6) bonds. The number of monosaccharides in an oligosaccharide is indicated by means of the DP-value ("Degree of Polymerisation"). A DP-value of 3 means that the oligosaccharide is composed of three monosaccharides. Oligofructoses are oligosaccharides consisting entirely of fructose units. When an oligosaccharide also comprises one or more glucose units these will be linked by means of an ~~α(1-2)~~ α-(1-2) bond to a fructose unit. The composition of oligosaccharides is also designated with the formula G_mF_n, wherein G represents glucose and

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F fructose and wherein m equals 0 or 1 and n is an integer larger than or equal to 0. Particularly suitable oligosaccharides are those wherein m equals 1 and n is 2 to 8, preferably 2 or 3.

Please amend the paragraph beginning at page 1, line 31, as follows:

Oligosaccharides can hardly be hydrolysed, if at all, in the human stomach and small intestine. It is known of oligofructose that the digestive enzymes of the human have no effect on the ~~β(2-1)~~ β-(2-1) and ~~β(2-6)~~ β-(2-6) bond in the molecule. They therefore pass through the stomach and the small intestine without being degraded and absorbed into the body. The oligosaccharides do not however leave the body but are metabolised by the microbial flora of the large intestine. Released herein in addition to gas are volatile fatty acids which in turn again serve as an energy source for the intestinal flora. This phenomenon explains why oligosaccharides have a lower energy value for humans than free sugars such as glucose, fructose and sucrose, which are absorbed into the body. Oligosaccharides do however have sufficient sweetening power to serve as sugar ~~substitute~~ substitutes.

Please amend the paragraph beginning at page 2, line 16, as follows:

Different oligosaccharides, which are prepared in diverse ways, are already commercially available ~~at the moment~~.

Please amend the paragraph beginning at page 2, line 19, as follows:

Oligosaccharides can be made by partial enzymatic hydrolysis of longer vegetable inulin chains. A method herefor is described for instance in [[the]] European patent application 440.074.

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Please amend the paragraph beginning at page 2, line 23, as follows:

Oligosaccharides can likewise be enzymatically synthesized. For this enzymatic production route use is made of enzymes, fructosyltransferases, which convert sucrose to a mixture of oligosaccharides and which are isolated from different micro-organisms ([[JP-]] see Japanese Patent 80/40193).

Please amend the paragraph beginning at page 2, line 28, as follows:

The known production routes have a number of drawbacks however. Firstly, ~~both~~ the known production methods are relatively expensive. In addition to the desired oligosaccharides with a chain length of 2 to about 7, in the produced mixture there also occur a comparatively large number of free sugars and oligosaccharides with a higher chain length. The drawback to many free sugars is that they result in an increase in the energy value of the mixture. Free sugars have for instance an energy content of 17 kilojoule per gram, while pure GF_2 and GF_3 have an energy content of 4 to 6 kilojoule per gram. In addition, free sugars cause dental decay (caries).

Please amend the paragraph beginning at page 3, line 6, as follows:

In contrast to some other sweeteners, such as for example Aspartame, oligosaccharides have good organoleptic properties.

Please add the following new section heading beginning at Page 3, between lines 11 and 12, as follows:

SUMMARY OF THE INVENTION

Please amend the paragraph beginning at page 3, line 32, as follows:

The particular advantage of the method according to the invention is that the chain length distribution is narrower, whereby no or few free sugars occur in the end product. The

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consequence hereof is a lower cariogenicity and the desired lower energy value. There also occur fewer oligosaccharides with a chain length of more than 5. The advantage hereof is that the oligosaccharides produced according to the invention have a higher specific sweetening capacity. It is the case that the sweetening capacity depends on the "average chain length". The higher the average chain length of a mixture, the lower the sweetening capacity. The advantage of a high specific sweetening capacity is that extra sweeteners hardly have to be added in processing of the product.

Please amend the paragraph beginning at page 4, line 7, as follows:

A similar consideration applies ~~in respect of~~ to solubility. It is also the case here that when the average chain length increases the solubility ~~deceases~~ decreases. The mixtures according to the present invention ~~therefore~~ have a higher solubility than [[the]] mixtures obtained by means of enzymatic synthesis or enzymatic hydrolysis. In addition, production costs are considerably reduced.

Please add the following new section heading and paragraphs beginning at page 4, between lines 18 and 19, as follows:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a photograph illustrating the oligosaccharide-producing activity of wild type and modified forms of the *Streptococcus mutans* fructocyltransferase incubated with sucrose and analyzed on TLC.

FIG. 2 is a photograph illustrating the results of TLC-analysis of transgenic tobacco plants which express the fructocyltransferase gene of *Streptococcus mutans*.

FIG. 3 is a photograph illustrating the SDS-PAGE gel of purified SST from onion seed.

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FIG. 4 is a photograph illustrating the results of TLC-analysis of the reaction products of purified SST from onion seed incubated with sucrose.

FIG. 5 is a graph of the protein elution profile (A280) and the fructocyltransferase activity of two isoforms of 6-SFT obtained from barley.

FIGS. 6A through 6D are chromatograms of 2 isoforms of 6-SFT obtained from barley.

FIG. 7 is a graph of the enzymatic activity of a pool of fractions of 6-SFT after isoelectric focusing under non-denaturing conditions.

FIG. 8 is a photograph illustrating the SDS-PAGE gel after two-dimensional analysis of pool II of Figure 6.

FIG. 9 is a photograph illustrating the two-dimensional gel electrophoreses of the IEF-markers phycocyanin, beta-lactoglobulin, and bovine carbonic anhydrase.

FIG. 10 is a schematic view of the strategy used to obtain the cDNA clone which codes for 6-SFT from barley.

FIG. 11A illustrates the relationship between Figures 11B and 11C.

FIGS. 11B and 11C show the amino acid sequence of 6-SFT from barley compared to different invertases, levanases and levansucrases.

FIG. 12 is a dandrogram of 6-SFT from barley with different invertases, levanases and levansucrases, based on derived immunoacid sequences.

FIG. 13 is a graph illustrating the functional expression of barley 6-SFT in *Nicotiana plumbaginifolia* protoplasts.

FIG. 14 is a graph illustrating the functional expression of barley 6-SFT in *Nicotiana plumbaginifolia* protoplasts.

FIG. 15 is a photograph illustrating a native IEF-GEL of a purified enzyme extract of FFT from *Helianthus tuberosus* L.

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FIG. 16 is an HPLC-diagram of tryptic digests of the FFT isoform T1.

FIG. 17 is an HPLC-diagram of tryptic digests of the FFT isoform T2.

Please insert a new section heading beginning at page 4, between lines 18 and 19, as follows:

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Please amend the paragraph beginning at page 4, line 19, as follows:

In order to select a gene which codes for an enzyme capable of converting sucrose into an oligosaccharide it is possible to search in any possible organism which contains fructosyltransferase activity, for instance micro-organisms such as bacteria, or plants. It is known of many micro-organisms that they contain fructosyltransferases which are capable of producing fructans from sucrose. These enzymes transfer fructose units from sucrose to a fructan acceptor molecule. Microbial fructosyltransferases normally produce fructans with a high DP. The use of a number of fructosyltransferases to manufacture transgenic plants for the production of such polysaccharides is already described in the literature. It is thus known that by incorporating the *Sac*B-gene of *Bacillus subtilis* in plants the fructan pattern of these plants can be modified (see International Publication No. WO 89/12386). This still relates however to the production of high-molecular polysaccharides.

Please amend the paragraph beginning at page 4, line 36, as follows:

Another gene which is known to code for a fructosyltransferase which can convert sucrose into high-molecular fructans is the *ftf* gene of *Streptococcus mutans*. According to the present invention, [[It]] it has now been found surprisingly that in addition to high-molecular weight fructans this fructosyltransferase also produces significant quantities of oligosaccharides

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in the trisaccharide class (1-kestose). Mutants have also been found which only accumulate trisaccharides and not polysaccharides.

Please amend the paragraph beginning at page 5, line 9, as follows:

A large number of other micro-organisms is likewise capable of fructosyltransferase production. These comprise, but are not limited[[,]] to, endospore-forming[[,]] rod bacteria and cocci (for example *Bacillus*), gram-positive cocci (for instance *Streptococcus*), gram-negative aerobic rod bacteria and cocci (for instance *Pseudomonas*, *Xanthomonas*, *Azotobacter*), gram-negative facultative anaerobic rod bacteria (for instance *Erwinia, Zymomonas*), actinomycetes (for instance *Actinomyces*, *Rothia*), and cyanobacteria (for instance *Tolypothrix tenius*).

Please amend the paragraph beginning at page 6, line 5, as follows:

Another example is the sucrose-fructan-6-fructosyltransferase (6-SFT) from barley (*Hordeum vulgare* L.). According to one embodiment of the invention, transgenic plants are provided which express [[the]] 6-SFT for the production of oligo-saccharides.

Please amend the paragraph beginning at page 7, line 30, as follows:

In a preferred embodiment of the invention the expression construct therefore also comprises a targeting sequence for directing the fructosyltransferase activity to one or more specific plant cell compartments. Examples of targeting sequences are the signal sequence and vacuolar targeting sequence of the carboxypeptidase Y (*cpy*) gene, that of patatine from the potato, [[or]] that of sporamine from the sweet potato, or the signal sequence and apoplastic targeting sequence of the pathogenesis-related protein S-gene (*pr-s*). These are examples, and the skilled person will ~~himself~~ be capable of selecting other targeting sequences.

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Please amend the paragraph beginning at page 8, line 3, as follows:

The expression construct can in ~~principe~~ principle be modified such that targeting takes place to any random cell compartment, such as the vacuole, plastides, cell wall, cytoplasm etc.

Please amend the paragraph beginning at page 8, line 17, as follows:

The oligosaccharides according to the invention can be used as ~~substitute~~ substitutes for sugar, glucose syrup and isoglucose in "light" versions of different food products. Examples of such food products are confectionery, biscuits, cakes, dairy products, baby food, ice cream and other desserts, chocolate and the like. The stimulation of Bifidobacteria is also important for the health of animals. The oligosaccharides according to the invention can therefore also be applied in for instance animal feed.

Please amend the paragraph beginning at page 8, line 26, as follows:

The present invention will be further elucidated on the basis of the examples hereinbelow, which are only given by way of illustration of the invention and are not intended to limit [[it]] the invention in any way. Reference is made in the examples to the annexed figures which show the following:

Please amend the paragraph beginning at page 8, line 31, as follows:

Figure 1 shows the oligosaccharide-producing activity of wildtype and modified forms of the *Streptococcus mutans* fructosyltransferase (*ftf*) which is incubated with sucrose and analysed on TLC as described by Cairns, A.J. and Pollock, C.J., New Phytol. 109, 399-405 (1988). Samples of cultures which were derived from colonies and purified proteins were incubated overnight with 200 mM sucrose in 50 mM sodium phosphate buffer with 1% Triton X-100 at 37°C. Lane 1 shows the reaction products of an *S. mutans* culture; lane 2 shows the activity of the purified enzyme from *S. mutans*; lane 3 shows the activity of an *E. coli* strain harbouring the

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plasmid pTS102; lane 4 shows the activity of an *E. coli* strain harbouring plasmid pTD2; and lane 5 shows the activity of an *E. coli* cell which is transformed with the mature *S. mutans* fructosyltransferase gene under the regulation of an *E. coli* promotor. The oligosaccharide standards used are in lane A, an extract of an *Allium cepa* bulb, and in lane H, an extract of a *Helianthus tuberosus* tuber. In the figure, F represents fructose, G represents glucose, S represents sucrose (disaccharide), N represents neokestose (F2-6G1-2F, trisaccharide), I represents 1-kestose (G1-2F1-2F, trisaccharide), and K represents kestose (G1-2F6-2F, trisaccharide). Higher molecular weight oligosaccharides (DP = 4-9) are likewise indicated.

Please amend the paragraph beginning at page 9, line 16, as follows:

Figure 2 shows the TLC-analysis of transgenic tobacco plants (KZ) which express the fructosyltransferase gene of *S. mutans*. Oligosaccharides accumulate in these plants. Lane H shows, as a control, an extract of a *Helianthus tuberosus* tuber.

Please amend the paragraph beginning at page 9, line 21, as follows:

Figure 3 shows the SDS-PAGE gel of purified SST from onion seed. A single band was visible in the SST sample on this gel, stained by means of silver-staining. M represents molecular weight markers wherein their size is indicated in kilodaltons (kD).

Please amend the paragraph beginning at page 9, line 26, as follows:

Figure 4 shows the reaction products of purified SST from onion seed which is incubated with sucrose (lanes 4 and 5: O-*in vitro*). Only trisaccharides are formed. Lane 1 shows the extract of tulip stalks (T), lane 2 shows the extract of *Helianthus tuberosus* tubers (H), and lane 3 shows the extract of an *Allium cepa* bulb (O). M represents monosaccharide, S represents sucrose (disaccharide), N represents neokestose (F2-6G1-2F, trisaccharide), and I represents

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1-kestose (G1-2F1-2F, trisaccharide). Higher oligosaccharides (DP4-5) are likewise indicated. The products were analysed on TLC as described for ~~figure~~ Figure 1.

Please amend the paragraph beginning at page 9, line 36, as follows:

~~Figure 5 shows~~ Figures 5 and 6A through 6D show the separation of 2 isoforms of the sucrose-fructan 6-fructosyltransferase (6-SFT) from barley after the second anion exchange chromatography step on a Resource Q column in a purification procedure. ~~Figure 5A~~ Figure 5 shows the protein elution profile (A280) and the fructosyltransferase activity of the fractions obtained after chromatography after incubation with 0.2M sucrose in 25 mM methylpiperazine (HCl) buffer (pH 5.75). [[De]] The chromatograms ~~(Fig. 5B)~~ (Figs. 6A through 6D) were obtained by pulsed amperometric detection after anion exchange HPLC separation on a CarboPack-PA100 column. The reaction products were obtained after incubation of pool I and pool III with sucrose alone, or sucrose and isokestose. The carbohydrates were identified by their retention times and trehalose was used as an internal standard.

Please amend the paragraph beginning at page 10, line 12, as follows:

Open circles in ~~fig. 5A~~ Figure 5 represent fructosyltransferase activity, which is indicated as the sum of formed kestose, bifurcose, isokestine and kestine. In ~~fig. 5B~~ Figures 6A through 6D, p corresponds with a non-identified product resulting from isokestose contaminants, and c with a contamination of the isokestose substrate.

Please amend the paragraph beginning at page 10, line 18, as follows:

Figure [[6A]] 7 shows a graph of the enzymatic activity of a pool of fractions of 6-SFT (referred to as pool II; see ~~figure 5~~ Figures 5 and 6A through 6D) after isoelectric ~~focussing~~ focusing under non-denaturing conditions. Closed triangles indicate beta-fructosidase activity measured as released fructose, while open circles indicate the fructosyltransferase activity

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measured as formed kestose. Figure [[6B]] 8 is an SDS-PAGE gel after two-dimensional analysis of pool II after the second anion exchange chromatography. The two 6-SFT isoforms are shown herein. Both isoforms are found to consist of two subunits of respectively 23 kDa and 49 kDa. Figure [[6C]] 9 is the two-dimensional gel electrophoresis of the IEF-markers phycocyanin (pI 4.6), beta-lactoglobulin (pI 5.1) and bovine carbonic anhydrase (pI 6.0).

Please amend the paragraph beginning at page 10, line 32, as follows:

Figure [[7]] 10 is a schematic view of the strategy used to obtain the cDNA clone which codes for 6-SFT from barley.

Please delete the paragraph beginning at page 10, line 34.

Please amend the paragraph beginning at page 10, line 36, as follows:

Figure [[9]] 11 is an overview of the derived amino acid sequence of 6-SFT from barley, different invertases (beta-fructosidases), levanases and levansucrases. The overview was produced with the Pileup program of the GCG sequence analysis software package. The following abbreviations were used:

Please amend the paragaraph beginning at page 11, line 4, as follows:

H.v. 6-SFT = sucrose-fructan 6-fructosyltransferase from barley[[.]];

Please amend the paragraph beginning at page 12, line 10, as follows:

Figure [[10]] 12 is a dendrogram of 6-SFT from barley with different invertases (beta-fructosidases), levanases and levansucrases, based on derived amino acid sequences. The dendrogram was generated with the sequences described in ~~figure 9~~ Figure 11 making use of the Pileup program of the GCG sequence analysis software package.

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Please amend the paragraph beginning at page 12, line 16, as follows:

~~Figure 11 shows~~ Figures 13 and 14 show the functional expression of barley 6-SFT in *Nicotiana plumbaginifolia* protoplasts. Error bars indicate the average standard deviation. The 6-SFT cDNA was expressed for 27 hours in protoplasts. Samples were taken a number of times and the fructosyltransferase activity was determined in protoplast extracts by incubation with sucrose (Fig. [[11A]] 13) or sucrose and isokestose (Fig. [[11B]] 14). Open circles show the enzyme activity of extracts of protoplasts which were transformed with the 6-SFT gene construct. Open squares show the activity of extracts of protoplasts transformed with the vector without the 6-SFT cDNA.

Please amend the paragraph beginning at page 12, line 27, as follows:

Figure [[12]] 15 is a native IEF-gel of a purified enzyme extract of fructan-fructan fructosyltransferase (FFT) from *Helianthus tuberosus* L.[[.]] After Coomassie Blue staining there can be seen, in addition to the two most important isoforms of the FFT (T1 (pI 4.45) and T2 (pI 4.75)), a band with a pI of approximately 5.5, which probably corresponds with denatured FFT.

Please amend the paragraph beginning at page 12, line 34, as follows:

Figures ~~13 and 14~~ 16 and 17 are HPLC-diagrams of tryptic digests of the FFT isoforms T1 (Fig. [[13]] 16) and T2 (Fig. [[14]] 17).

Please amend the paragraph beginning at page 13, line 5, as follows:

A large number of microbes [[was]] were screened for their capacity to produce oligosaccharides from sucrose. For this purpose bacteria cultures were grown overnight in a liquid nutrient. The oligosaccharide-producing activity was determined by incubating a sample of the culture with 200 mM sucrose in the presence of 0.1% Triton X-100. The reaction products

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were separated by means of TLC and made visible using a fructose-specific reagent (Cairns, A.J. and Pollock, C.J., New Phytol. 109, 399-405 (1988)). It was found as a result of this screening that *Streptococcus mutans* is an effective producer of oligosaccharides (see ~~figure~~ Figure 1). The oligosaccharide-producing enzymatic activity was purified from the *Streptococcus mutans* culture by means of DEAE-ion exchange chromatography and gel permeation chromatography. It was found herefrom that the enzymatic activity was caused by the product of the *ftf* gene previously described by Shiroza and Kuramitsu, (J. Bacteriol.[[,]] 170, 810-816 (1988)).

Please amend the paragraph beginning at page 13, line 22, as follows:

The fructosyltransferase (*ftf*) gene from plasmid pTS102 (Shiroza and Kuramitsu, *supra*) was subsequently cloned as an EcoRV-BglII fragment in the multiple cloning site of pEMBL9 (Dente et al., Nucl. Acids Res. 11, 1645-1655 (1983)) and expressed from the lacZ promotor present in this plasmid. *E. coli* was then transformed herewith. The bacteria was hereby made capable of producing oligosaccharides.

Please amend the paragraph beginning at page 14, line 1, as follows:

For mutagenesis of the *ftf* gene of *Streptococcus mutans*, the plasmid pTS102 was integrated into the genome of *Synechococcus* sp. PCC 7942 (R2-PIM9) by means of the genomic integration system (Van der Plas et al., Gene 95, 39-48 (1990)), which resulted in strain R2-PTS. This cyanobacteria R2-PTS strain expresses the fructosyltransferase gene. The R2-PTS strain is sucrose-sensitive due to polymer accumulation in the periplasm. An R2-PTS culture was mutated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which induces point mutations (T → C and G → A mutations). Mutants with a changed fructosyltransferase activity were selected. The culture mutated by means of MNNG was plated on sucrose-containing medium and a total of 400 sucrose-resistant colonies were tested for [[a]] changed fructosyltransferase activity.

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Please amend the paragraph beginning at page 14, line 16, as follows:

Derived from these colonies were R2-PTS cultures which were concentrated by means of centrifugation. The thus obtained pellets were resuspended in 50 mM sodium phosphate buffer with 1% Triton X-100, 200 mM sucrose and incubated overnight at 37°C. The reaction products were analysed by means of TLC-analysis (Cairns and Pollock, *supra*). The TLC was developed three times in 85:15 acetone:water and subsequently treated with atomized urea as described by Wise et al., Analytical Chemistry 27, 33-36 (1955). This method preferably stains fructose and fructose-containing polymers.

Please amend the paragraph beginning at page 14, line 27, as follows:

Of the mutants substantially producing trisaccharides, one was chosen for *in vitro* demonstration of the enzymatic oligosaccharide-forming activity of the mutated *ftf* gene in the above described manner.

Please amend the paragraph beginning at page 14, line 31, as follows:

According to the invention, other mutagenesis methods (site-directed or random) and genes which code for fructosyltransferases from other organisms can likewise be used to select a gene for a mutant oligosaccharide-producing protein.

Please amend the paragraph beginning at page 15, line 5, as follows:

A. Construction of 35S-*ftf*-NOS in a plant transformation vector.

Please amend the paragraph beginning at page 15, line 7, as follows:

The plasmid pMOG18 which contains a plant-specific 35S promotor with an enhancer duplication and sequences which stimulate the translations of mRNA is described by ~~Symons et al. (Bio/Technology 8, 217-221 (1990))~~ Symons et al., Bio/Technology 8, 217-221 (1990). It

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contains the 35S-promotor-uidA-gene-NOS-terminator construct. A pBluescript II SK-plasmid from Stratagene (San Diego, CA, U.S.A.), from which the internal BamHI-site was removed by digestion with BamHI and filling in the sticky ends with Klenow and ligating once again, was used for further cloning. The 35S-uidA-NOS-fragment was obtained by digestion with EcoRI and HindIII of pMOG18 and in this BamHI-pBluescript was cloned in the corresponding EcoRI/HindIII site, resulting in plasmid pPA2. Plasmid pPA2 was digested with NcoI and BamHI and the vector-containing fragment was isolated.

Please amend the paragraph beginning at page 15, line 26, as follows:

In order to obtain an NcoI location close to the mature processing site of the *ftf* gene (nucleotide position 783) (J. Bacteriol. 170, 810-816 (1988)), site-directed mutagenesis was performed as described by Kramer et al. (Nucleic Acids Res. 12, 9441-9456 (1984)) with the following oligonucleotide: 5'-GGCTCTCTTCTGTTCCATGGCAGATGAAGC-3' (SEQ ID NO:3). Resulting ~~herefrom~~ therefrom was plasmid pTD2. At amino acid position +1 (nucleotide position 783) relative to the mature processing site a glutamine was hereby changed into a methionine. The NcoI/PstI fragment, in which the sequence coding for the mature fructosyltransferase is present, was used for further cloning. From this plasmid, the *ftf* gene was isolated as an NcoI/PstI fragment and this fragment was ligated in the pPA2 vector-containing fragment described above. This results in plasmid pTX. pTX contains the 35S-*ftf*-NOS-fragment in which *ftf* shows the mature fructosyltransferase gene without its signal sequence region. pTX was digested with XbaI and HindIII, the fragment containing the complete construct (35S-*ftf*-NOS) was cloned in the XbaI/HindIII restriction site of pMOG23 (Symons et al., *supra*) a derivative of the binary plant vector pBIN19 (Bevan, Nucl. Acids. Res. 12, 8711-8721). This resulted in plasmid pTZ.

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Please amend the paragraph beginning at page 16, line 11, as follows:

B. Manufacture and analysis of transgenic plants which express the mature *ftf* gene.

Please amend the paragraph beginning at page 16, line 13, as follows:

The pTZ-plasmid was conjugated in *Agrobacterium tumefaciens* LB4404 (Hoekema et al., Nature 303, 179-180 (1983)) in a three-point crossbreeding making use of the helper plasmid pRK2013 (Lam, Plasmid 13, 200-204 (1985)). The construct was introduced into *Nicotiana tabacum* var. Petit Havanna (SR1) using the leaf disc transformation method (Horsch et al., Science 227, 1229-1232 (1985)). The regenerated plants were called KP-plants and were selected for kanamycine resistance and cultured on MS medium (Murashige and Skoog, Physiol. Plant. 15, 473-497 (1962)). Thereafter, the plants were grown on soil in the greenhouse and analysed.

Please amend the paragraph beginning at page 15, line 25, as follows:

The leaf material was cut off and ground in an eppendorf tube. After centrifugation (2 minutes at 16,000 rpm), 1 μl supernatant was analysed on TLC as described in ~~example~~ Example 1.

Please amend the paragraph beginning at page 16, line 29, as follows:

Oligosaccharides were never found in wildtype plants or in plants which were transformed with non-related constructs. The screening of the transformants demonstrated oligosaccharide-accumulating plants using this method (see ~~figure~~ Figure 2). The expression levels varied between individual plants which were transformed with the same construct. This is a normal phenomenon in transformation experiments in plants. The variation of the expression levels depends substantially on the integration position in the genome (position effect).

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Please amend the paragraph beginning at page 17, line 3, as follows:

Oligosaccharide-producing enzyme (SST) from the onion.

In addition to the above-used fructosyltransferase genes originating from micro-organisms, such enzymes are also produced by plants. In this example, the SST gene from onion seed is used.

Please amend the paragraph beginning at page 17, line 8, as follows:

The SST protein from onion seed was purified by chromatographic procedures making use of the following ~~protocol. The~~ protocol: the seed was incubated at 22°C between moist cloths for 2 to 3 days and homogenised in 50 mM phosphate-citrate buffer with a pH of 5.7. The starch and debris were centrifuged off at about 10,000 g for 10 minutes. Ammonium sulphate was added to the supernatant to 20%, and the precipitate collected by centrifugation. The concentration of ammonium sulphate in the supernatant was increased to 80%, and the precipitate collected and dissolved in 20 mM NaAc pH 4.6. The solution was dialysed overnight with three buffer changes (20 mM NaAc) and the solution was clarified by centrifugation. The supernatant was placed on an FPLC monoS-column and ~~eluated~~ eluted in 20 mM NaAc pH 4.6 with a 0-0.5 M NaCl gradient. After dialysis overnight against 10 mM NaAc pH 5.6, the solution was placed onto a raffinose-epoxy sepharose column (Pharmacia), which was equilibrated in 10 mM NaAc pH 5.6. Elution took place with a linear gradient consisting of 10 mM NaAc pH 5.6 (buffer A) and 10 mM phosphate-citrate buffer, pH 7.0, plus 0.5 M NaCl-buffer (buffer B). The active fractions were dialysed overnight against 20 mM phosphate-citrate [[-]] buffer, [[Ph]] pH 7.0, and placed on a monoQ FPLC-column in 20 mM phosphate-citrate buffer, pH 7.0. The column was ~~eluated~~ eluted with a gradient of 0-0.5 M NaCl. For a final purification the protein was placed onto a Sepharose 6-column and ~~eluated~~ eluted with 50 mM phosphate buffer, pH 6.5, with 1% Triton X-100. The silver staining of an SDS-PAGE gel of

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purified SST from onion seed revealed only one band with a molecular weight of approximately 68,000 d (see ~~figure~~ Figure 3).

Please amend the paragraph beginning at page 18, line 1, as follows:

When this purified SST was incubated with sucrose, only 1-kestose was produced. No significant invertase activity was observed (see ~~figure~~ Figure 4).

Please amend the paragraph beginning at page 18, line 4, as follows:

The amino acid sequence of the purified protein was determined on the basis of peptides obtained by gradual breakdown. On the basis of this information PCR-probes were designed with which the gene coding for the SST of onion seed was isolated. In the same manner as described in ~~examples~~ Examples 1 and 2, it was ~~hereby~~ demonstrated both *in vitro* and *in vivo* that the gene codes for an enzyme capable of producing oligosaccharides.

Please amend the paragraph beginning at page 18, line 14, as follows:

Applicability with other plant species.

In order to illustrate the general applicability of the technology, the *ftf* construct described in ~~example~~ Example 2 was introduced into different crops. The potato was thus transformed according to the method described in Visser, Plant Tissue Culture Manual B5, 1-9, Kluwer Academic Publishers, 1991. The resulting transgenic plants accumulated oligosaccharides in each tested organ. The same construct was also introduced into the beet (*Beta vulgaris* L.), which was transformed as described by D'Halluin et al., Biotechnology 10, 309-314 (1992). The resulting transgenic beet plants accumulated significant quantities of oligosaccharides in, for instance, their leaves and roots. The same constructs were introduced into *Brassica napus* L. which was transformed as according to Block et al., Plant Physiol. 91, 694-701 (1989). The resulting transgenic plants accumulated significant levels of oligosaccharides in, for instance,

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their leaves and storage organs. It is of course not essential that the plants are transformed in the manner indicated. Other methods within the reach of the skilled person can also be used.

Please amend the paragraph beginning at page 19, line 17, as follows:

Sucrose-fructan 6-fructosyltransferase (6-SFT) from barley.

1. Introduction.

Sucrose-fructan 6-fructosyltransferase (6-SFT) is a key enzyme for the biosynthesis of branched fructans (also called graminans) which are typical for grasses. The enzyme forms kestose from sucrose and bifurcose from sucrose and isokestose. In this example the purification of a 6-SFT from barley (*Hordeum vulgare* L.) is described, in addition to the cloning of the full cDNA and confirmation of the functionality.

Please amend the paragraph beginning at page 19, line 29, as follows:

2. Purification of sucrose-fructan 6-fructosyltransferase.

Primary leaves of eight- to ten-day-old barley plants (*Hordeum vulgare* L. cv Express) were cut off and exposed to light continuously for 48 hours to induce the accumulation of fructans and [[the]] enzymes of the fructan biosynthesis, as described by Simmen et al., Plant Physiol. 101, 459-466 (1993). The leaves were subsequently frozen in liquid nitrogen and stored at -70 °C until they were used.

Please amend the paragraph beginning at page 20, line 11, as follows:

The enzyme solution was purified by means of affinity chromatography on Blue Sepharose. For this purpose the enzyme solution was filtered through a 0.45 micrometer Millipore filter and loaded at a flow speed of 2 ml per minute on a column (26x120mm) of Blue Sepharose-6-fast flow (Pharmacia, Uppsala, Sweden), which had previously been equilibrated with the above described dialysis buffer. In order to remove proteins without affinity for the

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dye, the column was washed with three bed volumes of the dialysis buffer. Bound proteins were eluated at a flow speed of 3 ml per minute (5 ml fractions), first with 0.2 M NaCl in 10 mM methylpiperazine (HCl) buffer (pH 5.75) for 30 minutes, followed by a linear gradient of 0.2 M to 0.5 M NaCl in the same buffer within 90 minutes.

Please amend the paragraph beginning at page 20, line 25, as follows:

All fractions which contained 6-SFT activity were pooled, dialysed overnight at 4°C against dialysis buffer, and then concentrated to one third of the starting volume by covering the dialysis bag with polyethylene glycol 40,000 and incubating it for 4 hours at 4°C.

Please amend the paragraph beginning at page 20, line 30, as follows:

For a first anion exchange chromatography step the 6-SFT fraction was filtered and loaded at a flow speed of 3 ml per minute on a 6 ml ~~resource~~ Resource Q column (Pharmacia), which had been equilibrated earlier with dialysis buffer. After the column was washed with 10 mM methylpiperazine (HCl) buffer (pH 5.75), the bound protein was ~~eluated~~ eluted with a linear gradient of 0 to 0.15 M NaCl in the same buffer within 8 minutes at a flow speed of 15 ml per minute. Fractions of 1 ml were collected. The fractions which contained 6-SFT were pooled and supplemented with ammonium sulphate to a final concentration of 2 M.

Please amend the paragraph beginning at page 21, line 27, as follows:

For a second anion exchange chromatography step the desalted sample was loaded onto a 6 ml Resource Q column (Pharmacia). The conditions and buffers were the same as for the first anion exchange chromatography step but the fraction, size was reduced to 0.5 ml. The fractions which contained 6-SFT activity were combined in pool I, II and III (~~fig. 5A~~ FIG 5).

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Please amend the paragraph beginning at page 22, line 14, as follows:

Neutral carbohydrates were analysed by means of anion exchange chromatography on a CarboPac PA-100 column (Dionex, Sunnyvale, California, USA) with a Dionex DX-300 gradient chromatography system coupled to pulsed amperometric detection (Simmen et. al., *supra*). Prior to analysis by means of anion exchange chromatography, enzyme activities freeing glucose from sucrose were detected in the fractions collected during the enzyme purification[[, this]] using the glucose test kit (GOD-Perid method, Boehringer GmbH, Mannheim, Germany) in accordance with the instructions of the manufacturer.

Please amend the paragraph beginning at page 22, line 24, as follows:

Two 6-SFT isoforms with indistinguishable catalytic properties were isolated by the purification (~~table~~ see Table I). By affinity chromatography on the HighTrap blue column and by hydrophobic interaction chromatography on the alkylsuperose column, the invertase (beta-fructosidase) activity was almost completely separated from the 6-SFT. This means that 6-SFT has no invertase activity. The mol ratio between beta-fructosidase and fructosyltransferase activity fell by a factor of 6 after affinity chromatography and was then further reduced to a final ratio of approximately three after hydrophobic interaction chromatography ([[table]] see Table I). The remaining beta-fructosidase activity could not be separated from 6-SFT and therefore appears to be one of its catalytic properties.

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Please amend the paragraph beginning at page 22, line 37, as follows:

As already demonstrated by Simmen et al. ~~(supra)~~ *supra*, its capacity to transfer fructose to either sucrose or to isokestose is a characterizing property of 6-SFT. Both 6-SFT isoforms which were obtained after the second anion exchange column have the same catalytic properties as shown by HPLC-analysis of the products formed after incubation with sucrose alone or with sucrose and isokestose (~~fig. 5B~~ FIGS. 6A through 6D). In the presence of sucrose as the only substrate, mainly kestose is formed but sucrose is likewise hydrolysed to glucose and fructose. After incubation with sucrose and isokestose, mainly bifurcose is formed and much less sucrose is hydrolysed. This indicates that isokestose is the preferred acceptor compared with sucrose and that the beta-fructosidase activity is a component of the 6-SFT.

Please amend the paragraph beginning at page 23, line 14, as follows:

3. Gel electrophoresis.

To illustrate the purity of the two 6-SFT isoforms fractions of the Resource Q chromatography lying between the two 6-SFT peaks, and therefore containing both fractions, were pooled (pool II in ~~fig. 5~~ FIGS. 5 and 6A through 6D) and analysed by non-denaturing IEF gel-analysis combined with either an enzyme activity assay (~~fig. 6A~~ FIG. 7) or with SDS-PAGE analysis (~~fig. 6B~~ FIG. 8).

Please amend the paragraph beginning at page 23, line 26, as follows:

The 1 mm tubular gels were subsequently either cultured for 30 minutes in 5x sample buffer and loaded onto a 7.5-12% SDS polyacrylamide gel for a separation in the second dimension (Laemmli, Nature 227, 680-695[[,]] (1970)), or washed three times for ten minutes in 0.5 M citric acid Na_2HPO_4 buffer (pH 5.75) and cut into pieces of 2.5 mm for an enzyme activity assay. The 2.5 mm gel pieces were incubated in 0.4 M citric acid Na_2HPO_4 buffer (pH

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5.75) with 0.2 M sucrose and 0.02% NaN_3 for 12 hours at 27°C. After centrifugation at 13,000 g for 5 minutes the supernatant was collected, heated to 95°C for 3 minutes, supplemented with trehalose (internal standard, final concentration 0.1 μg/μl) and stored at -20°C for further analysis.

Please amend the paragraph beginning at page 24, line 3, as follows:

The two isoforms were clearly separated and both had [[a]] fructosyltransferase and likewise a beta-fructosidase activity. Their pI differed only slightly and was close to pH 5.0. After denaturation both 6-SFT isoforms provided on SDS-PAGE two subunits of respectively 49 and 23 kDa. This data and the almost complete identicity of the fragment patterns obtained by tryptic digestion (data not shown) indicate that the two isoforms display [[very]] many similarities in ~~respect of~~ structure and sequence. The negatively loaded 6-SFT (containing both isoforms) had a molecular weight of approximately 67 kDa as determined by size-exclusion chromatography (data not shown).

Please amend the paragraph beginning at page 24, line 16, as follows:

4. Determination of the N-terminal amino acid sequence.

Please amend the paragraph beginning at page 24, line 30, as follows:

The peptide sequence of the N-terminus of the 49 kDa subunit was determined and both, the large and the small, subunits were digested with trypsin in order to obtain internal peptide sequences. For both subunits two amino acid sequences of tryptic peptides were determined and used to design DNA primers (~~fig. 7~~ FIG. 10).

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Please amend the paragraph beginning at page 25, line 1, as follows:

5. Design of a probe.

A 397 bp fragment was generated by reverse transcription polymerase chain reaction (RT-PCR). For this purpose single-strand cDNA was synthesized by reverse transcription of Poly(A*)-RNA making use of a synthetic oligo-d(T) primer (23mer) and M-MuL V reverse transcriptase. PCR was performed according to the Perkin-Elmer protocol between the two synthetic, degenerated primers:

(i) CGCCTGCAGGTACCACATGTT(C/T)TA(C/T)CA(A/G)TA(C/T)AA(C/T)CC (SEQ ID NO:4); and

(ii) CCACGTCTAGAGCTCTC(A/G)TC(A/G)TACCA(A/C/G)GC(C/G)GTCAT (SEQ ID NO:5).

These primers were designed in accordance with two part sequences of peptides obtained after tryptic digestion of 6-SFT. The resulting PCR product was cloned in the pCR-II™ vector (TA-cloning kit, Invitrogen). Labelling of the fragment with α-^{32}p-dATP was performed with a random primed labelling kit (Boehringer GmbH, Mannheim, Germany) according to the instructions of the manufacturer.

Please amend the paragraph beginning at page 25, line 19, as follows:

6. Screening of a cDNA library.

The fragment of 397 bp generated [[as]] according to the method ~~under 5.~~ of paragraph 5 above was used as a probe in an RNA gel blot analysis of primary leaves, in which the accumulation of fructans was induced by continuous exposure to light for different tunes. There was found to be no hybridisation signal in the case of untreated leaves while a hybridising band of approximately 1800 bp accumulated rapidly in a manner which corresponded with the

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increase in 6-SFT activity in the leaves (data not shown). This result points to the presence of a messenger RNA of about 1800 bp in length.

Please amend the paragraph beginning at page 25, line 33, as follows:

To this end a cDNA expression library was first manufactured by extracting total RNA from 8 day-old cut primary leaves in which the synthesis of fructans was induced by continuous exposure to light for 48 hours. The leaves were ground in liquid nitrogen to a fine powder and suspended in RNA extraction buffer (0.1 M Tris (HCl), pH 9, with 10mM EDTA, 0.1 M NaCl and 25 mM DTT). The still frozen sample was further ground until a cream-like consistency was reached and the sample was then extracted with phenol-chloroform-isoamylalcohol (25:24:1;v:v:v) (Brandt and Ingversen, Carlsberg Res. ~~Commum.~~ Commun. 43, 451-469[[,]] (1978)). The method was modified somewhat by omitting a second homogenisation step and by precipitating the RNA overnight with 2M LiCl at 4°C after the last chloroform extraction. After a final ethanol precipitation poly (A)*-RNA was isolated by poly(U)-Sepharose chromatography (Brandt and lngversen, *supra*) and used for cDNA synthesis (ZAP-cDNA synthesis Kit, Stratagene, LaJolla, [[Ca]] California, USA).

Please amend the paragraph beginning at page 26, line 30, as follows:

After the first screening, 9 positive clones were isolated. After a further screening, 7 clones remained positive. Of these the sequence was partially determined from the 5' terminus and from the internal primers which were designed on the basis of the PCR product. All 7 clones appeared to code for the same protein, and four of them comprised the complete coding sequence. Of one of the possible clones of full length the sequence was wholly determined on both strands and it was found that it coded for a polypeptide which contained the 49 kDa subunit as well as the 23 kDa subunit (~~fig. 7~~ FIG. 10).

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Please amend the paragraph beginning at page 27, line 3, as follows:

~~A schematic view of the~~ The complete nucleotide sequence of the fully sequenced cDNA ~~is shown in fig. 8 . It~~ (SEQ. ID NO:1) comprises one long open reading frame which begins at nucleotide 46 and ends at nucleotide 1923 for two stop codons. The open reading frame codes for a polypeptide chain of 626 amino acids including a leader sequence of 67 residues in length.

Please amend the paragraph beginning at page 27, line 9, as follows:

The mature 6-SFT starts at nucleotide 246 and therefore has at least 559 amino acid residues with a calculated molecular weight of 61.3 kDa and a calculated pI of 5.37. All 5 of the partial amino acid sequences obtained from the purified protein are present in the amino acid sequence (SEQ ID NO:2) derived from the cDNA ~~(fig. 8)~~. The cDNA likewise contains 45 bp of a 5' non-translated and 171 bp of a 3' non-translated sequence with a poly(A) tail. A possible translation initiation signal (ATG) of the 6-SFT cDNA is localized at the nucleotide positions 46 to 48 and a possible polyadenylating sequence is present at the nucleotide positions 1973 to 1979. It has been found that the mature 6-SFT displays alpha-methyl-mannoside-reversible binding on ConA-Sepharose, which indicates that it is a glycoprotein (data not shown). Similarly, the derived amino acid sequence contains 6 possible glycosylating positions (Asn-X-Ser/Thr).

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Please amend the paragraph beginning at page 27, line 32, as follows:

In order to study the possible relation of the cDNA to known beta-fructosidases and fructosyltransferases, the derived amino acid sequence was compared with the sequence of different vegetable, fungal and bacterial invertases, and with bacterial levanases and levansucrases (~~fig. 9 and fig. 10~~ FIG. 15 and FIG. 16). The cDNA described herein has the highest homology with soluble acid invertases of the green soya bean (mungbean) (Arai et al., *supra*), carrot (Unger et al., *supra*), and tomato (Elliott et al., *supra*), and equally clear homologies with invertases, levanases and levansucrases from other kingdoms, that is, with a number of beta-fructosidases. The comparison of the amino acid sequence indicates at least five well conserved domains. Domains I (SEQ ID NO:6) and IV (SEQ ID NO:7) are less conserved between invertases and levansucrases than domains II (SEQ ID NO.:8), III (SEQ ID NO:9) and V (SEQ ID NO:10). With these enzymes domain III in particular is very conserved. Surprisingly, the most limited homology is that with bacterial levansucrases, that is, with a class of enzymes which catalyse a similar 6-fructosyl transfer reaction as 6-SFT (see the dendrogram in ~~fig. 10~~ FIG. 16).

Please amend the paragraph beginning at page 28, line 14, as follows:

7. Expression of 6-SFT in *Nicotiana plumbaginifolia* protoplasts.

The 6-SFT cDNA clone was sub-cloned in a derivative of the pUC119 plasmid vector (~~Samsbrook~~ Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring ~~Harbor,~~ Harbor (1989)) under the regulation of the expression signals of the cauliflower mosaic virus 35S transcript ([[see]] Neuhaus et al., Proc. Natl. Acad. Sci. USA 88, 10362-10366 (1991)).

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Please amend the paragraph beginning at page 28, line 23, as follows:

Protoplasts of *Nicotiana plumbaginifolia* were isolated and transformed largely as described by Goodall et al. (Meth. Enzymol. 181, 148-161 (1990)). In summary, 10 µg of the plasmid containing the 6-SFT cDNA was dispersed in a volume of 10 µl TE buffer in sterile 15 ml plastic tubes. Control transformations were carried out with 10 µg of the same plasmid without insert. 1 x 10^6 protoplasts were added up to a volume of 0.5 ml and mixed carefully with an equal volume 20% (w/v) polyethylene glycol 6000. After 2-5 minutes 6.5 ml K3 medium was added and the protoplasts incubated for two hours at 27°C. They were thereafter diluted 1:1 with the W5 osmoticum and pelleted for 10 minutes at 1000 g. All protoplasts (except those which were taken as control at t = 0 hour) were resuspended in 2 ml K3 medium and incubated at 27°C. After 3, 6, 9, 18 and 27 hours samples were taken for product analysis. The protoplasts were herein pelleted for 10 minutes at 1000 g after addition of 2 ml W5 osmoticum. The protoplast pellet was resuspended in 0.1 M citric acid Na_2HPO_4 buffer (pH 5.75), transferred to sterile Eppendorf tubes and frozen in liquid nitrogen. After defrosting the samples were vortexed, and cell debris was pelleted at 13.000 g for 3 minutes. The supernatants (50 to 100 µl) were desalted by guiding them over Biogel P-10 columns as described above. Desalted enzyme samples were incubated with 0.1 M sucrose or with 0.1 M sucrose in combination with 0.1 M isokestose in 50 mM citric acid Na_2HPO_4 buffer (pH 5.75) with 0.02% NaN_3 for 20 hours at 27°C. The product analysis was performed as described in the case of ~~Fig. 5~~ FIGS. 5 and 6A through 6D after stopping of the reaction by heating the samples at 95°C for 3 minutes.

Please amend the paragraph beginning at page 29, line 15, as follows:

After an initial lag-phase of about 3 hours extracts of protoplasts formed kestose from sucrose and bifurcose from sucrose and isokestose. This confirms that the cDNA codes for a

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functional 6-SFT (~~fig. 10~~ FIG. 12). Like the purified enzyme, the activity present in the protoplasts catalysed the production of bifurcose from sucrose and isokestose at a speed that was roughly four times higher than the production of isokestose from sucrose. These results confirm that the cDNA codes for a 6-SFT.

Please amend the paragraph beginning at page 29, line 35, as follows:

The purified enzyme was separated an a native IEF-gel and blotted on a PVDF membrane. The membrane was stained by means of a Coomassie Blue staining and the two most important FFT isoforms (respectively T1 and T2) were cut out (see ~~figure 12~~ FIG. 15).

Please amend the paragraph beginning at page 30, line 3, as follows:

Both proteins T1 and T2 were digested with trypsin and the peptides were separated by means of HPLC. The HPLC-diagrams of the digested FFT isoforms exhibit identical patterns (see ~~figures 13 and 14~~ FIGS. 16 and 17). The amino acid sequence was determined of two of the purified peptides of T2 (fractions 18 and 24). The sequence of the first peptide was:

NH_2 - E - Q - W - E - G - X - F - M - Q - Q - Y - X - X - (SEQ ID NO:11).

The other peptide had the following amino acid sequence:

NH_2 - A - V - P - V - X - L - X - X - P - L - (F/L) - I - X - W - V - (SEQ ID NO.12).

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Please amend the paragraph beginning at page 30, line 13, as follows:

In the same manner as in ~~example~~ Example 5 the cDNA was isolated and the sequence determined. Using a complete cDNA-clone plant cells were transformed to obtain transgenic plants.

Please amend the paragraph beginning at page 30, line 35, as follows:

The milk powder is dissolved in the water. The whole is heated to 40-45°C. The remaining dry ingredients are mixed and dissolved in the warm milk. The melted butter is then added. This whole is then pasteurised for 10 minutes at 72°C. The mixture is thereafter homogenised in a two-stage homogenizer at 150/35 bar. The ~~thus obtained~~ ice mix obtained is cooled rapidly to 5°C and the whole is subsequently left to mature for a minimum of 4 hours at 5°C. Finally, the ice mix is aerated and frozen to an overrun of 100%.

Please amend the paragraph beginning at page 31, line 18, as follows:

A syrup was produced from the oligosaccharides by heating, which syrup was mixed with the other ingredients. The bars were formed from the thus obtained mixture in a cylindrical press. Due to the omission of natural sugar the bar is much ~~lower-calory~~ lower calorie than [[the]] conventional bars.

A replacement abstract showing the change made is appended hereto as a separate page. No new matter has been added.

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